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Short communication

# High prevalence of *Hepatozoon*-infection among shepherd dogs in a region considered to be free of *Rhipicephalus sanguineus*

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ABSTRACT

Blood samples and ticks were collected from 100 shepherd dogs, 12 hunting dogs and 14 stray dogs in southern Hungary, in order to screen them for the presence of *Hepatozoon* spp. by PCR. Out of 126 blood samples, 33 were positive (26%). Significantly more shepherd dogs (31%) were infected, than hunting (8%) and stray dogs (7%). Three genotypes of *Hepatozoon canis* were identified by sequencing, differing from each other in up to six nucleotides in the amplified portion of their 18S rRNA gene. In *Dermacentor marginatus* larvae/nymphs and *Dermacentor reticulatus* nymphs, *H. canis* was present only if they had been collected from PCR-positive dogs, and the genotypes were identical in the ticks and their hosts. However, two *Haemaphysalis concinna* nymphs removed from a PCR-negative dog were found positive for *H. canis*, and the genotype detected in specimens of this tick species differed from that in the blood of their respective hosts. These results indicate that canine hepatozoonosis may be highly prevalent in regions where *Rhipicephalus sanguineus* is considered to be non-endemic. In addition, *H. canis* was identified for the first time in Hungary, as well as in *D. marginatus*, *D. reticulatus* and *Ha. concinna* ticks. Canine hepatozoonosis was significantly more prevalent west of the Danube river (where higher densities of red fox and golden jackal populations occur), suggesting a role of wild carnivores in its epidemiology.



## 1. Introduction

*Hepatozoon* spp. (Eucoccidiorida: Hepatozoidae) are unicellular, apicomplexan parasites belonging to the group of haemogregarines. They parasitize vertebrates as hosts and are transmitted by blood sucking arthropod vectors. Representatives of the genus infecting dogs occur both in the New and the Old World. American canine hepatozoonosis is caused by *Hepatozoon americanum*, affecting primarily striated muscles (Baneth et al., 2003). *Hepatozoon canis* is geographically more widespread – including the Americas, the tropical and temperate zone of Europe, Africa, Asia – and infects haemolymphatic tissues (spleen, liver, bone marrow, and lymph nodes) of dogs. Although the latter condition may frequently be asymptomatic, it can also result in severe, eventually fatal debilitating disease with anaemia, lethargy and cachexy (Baneth et al., 2003). An important aspect of the life cycle of both *Hepatozoon* spp. is that

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sporozoites, infective for the host, remain enclosed within oocysts in the body cavity of the relevant hard tick vector, therefore cannot be inoculated during blood feeding and the tick has to be eaten for the infection to establish. Wild carnivores are also susceptible (Gabrielli et al., 2010), and may play a role in the epidemiological cycle of canine hepatozoonosis.

The geographical distribution of *H. canis* in Europe is restricted to southern, Mediterranean and Balkan countries, where its typical vector, *Rhipicephalus sanguineus* is endemic. In other parts of the continent canine hepatozoonosis is diagnosed seldom, and usually in dogs with a history of travelling to the south (Deinert et al., 1997). However, there are reports of *H. canis* even from northern Europe, where it was detected in animals that never left their homeland. Such unique cases are exemplified by data from Ireland (Maguire et al., 2010) and Poland (under GenBank accession number EU165370).

In Hungary so far *Hepatozoon* infection was described only in rodents (Sebek, 1978). Since low prevalence of canine hepatozoonosis has been recently reported in Croatia close to the southern border of Hungary (Vojta et al., 2009; Dezdek et al., 2010), it was decided to evaluate three special groups of dogs – that are more likely to encounter potential vectors and wild animal reservoirs of *Hepatozoon* spp., than pet dogs – and their hard ticks, in order to see if canine hepatozoonosis has spread northward and is an emerging disease in the country.

## 2. Materials and methods

EDTA-anticoagulated blood samples were obtained by cephalic venipuncture from randomly selected 100 shepherd dogs, 12 hunting dogs and 14 stray dogs on 24 locations in south Hungary, during mid-summer of 2012. All ticks were removed from the dogs, and were immediately put into and stored in 70% ethanol. Data of dogs (sex, age) were recorded, but none of the animals were clinically evaluated. Blood smears were prepared on the day of sample collection (fixed, then stained with Giemsa), and the remainders of blood were frozen at −20 °C until further processing.

DNA was obtained with the QIAamp Mini Kit (QIAGEN, Hilden, Germany) – individually from all blood samples (200 μl) and ticks, including extraction controls – following the manufacturer's instruction. Eighty-four ticks were selected for molecular evaluation (i.e., at least one and a maximum of 12 from each infested animal). Prior to DNA extraction all ticks were sequentially washed in detergent containing water, in tap water and in distilled water. Air-dried specimens were minced with pointed scissors at the bottom of Eppendorf tubes, in 100 μl of phosphate-buffered saline (PBS), then subjected to an overnight digestion step (incubation at 56 °C for at least 8 h) with tissue lysis buffer and Proteinase-K.

The species of *Dermacentor* nymphs were identified molecularly (due to their morphological similarity to each other) by two PCRs which amplify fragments of the 16S and 12S rDNA genes (Black and Piesman, 1994; Norris et al., 1999). PCR products were resin purified (Wizard, Promega) and sequenced (Secugen S.L., Madrid, Spain).

For the presence of *Hepatozoon* spp. DNA samples were analyzed by a conventional PCR modified from Inokuma et al. (2002). In particular, the primers HepF 5′-ATA CAT GAG CAA AAT CTC AAC-3′ and HepR 5′-CTT ATT ATT CCA TGC TGC AG-3′ were used to amplify a 666 bp long fragment of the *Hepatozoon* 18S rRNA gene. 2.5 μl of extracted DNA were added to 22.5 μl of reaction mixture containing 1.0 U HotStart Taq DNA Plus polymerase (5 U/μl), 200 μM dNTP, 10 pmol of each primer, 2.5 μl of 10× Coral Load PCR buffer (15 mM $MgCl_2$ included) and 1.5 mM $MgCl_2$. Amplification was performed in a T-personal thermal cycler (Biometra, Goettingen, Germany). An initial denaturation step at 95 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 57 °C for 40 s and extension at 72 °C for 60 s. Final extension was performed at 72 °C for 7 min. DNA of a rodent *Hepatozoon* sp. served as positive control. PCR products were electrophoresed in 1.5% agarose gel (100 V, 30 min), stained with ethidium-bromide and visualized under ultra-violet light. Purification and sequencing was done – from 21 PCR-positive blood and 32 tick samples – by Macrogen Inc. (Korea). Representative sequences were submitted to the GenBank (accession numbers KC509526–KC509532).

Parasitaemia was evaluated from blood smears at three levels (<1%, 1–5%, and 5%<), based on counting *Hepatozoon* gamonts in 300 neutrophils and expressed as the percentage of infected cells. Exact confidence intervals (CIs) for prevalence rates at the 95% level were calculated according to Sterne's method (Reiczigel, 2003). Sample prevalence data were analyzed by using Fisher's exact test. The mean ages of animals were compared with Student's *t*-test. Differences were regarded significant when $P < 0.05$.

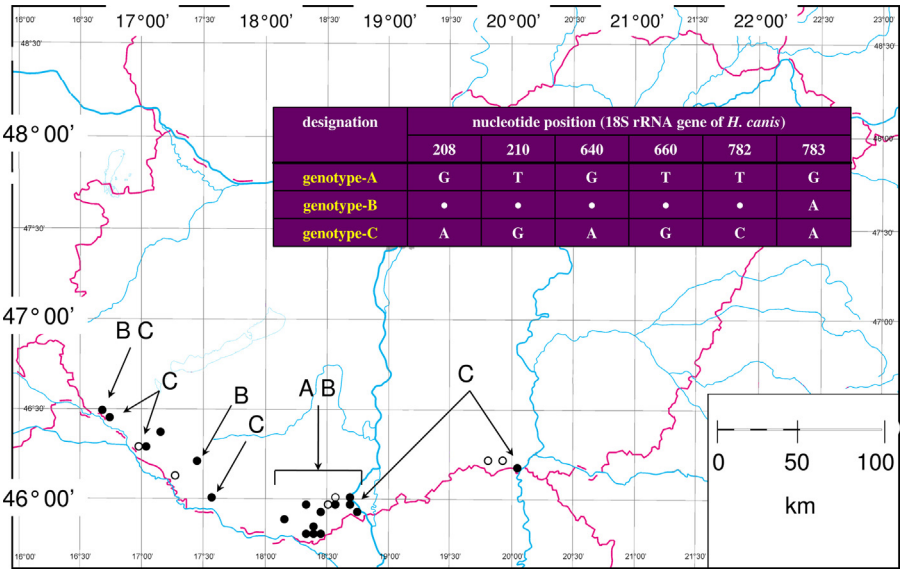
## 3. Results

### 3.1. Evaluation of blood samples

Out of 126 blood samples 33 were positive in the PCR for *Hepatozoon* spp. (26%, CI: 19–35%). The prevalence of *Hepatozoon* infection was 31% (31 out of 100, CI: 22–41%) among shepherd dogs, 8% among hunting dogs (1 out of 12, CI: 0–38%) and 7% among stray dogs (1 out of 14, CI 0–34%). This means that significantly more shepherd dogs were infected, than hunting and stray dogs ($P = 0.02$). Twenty-eight PCR-positive dogs had low levels of parasitaemia (<1%), indicating chronic (carrier) status. In further four animals the parasitaemia was moderate (1–5%), and only in one was it high (5%<). All except one positive samples originated from the south-western part of Hungary, which suggests that canine hepatozoonosis was significantly more prevalent west of the Danube river (Fig. 1).

Although male dogs were more frequently PCR-positive, than females (21 vs. 12), this was a non-significant association. Two (the youngest) PCR-positive dogs were only 6 months old. However, the mean age of *Hepatozoon*-infected dogs (4.03 ± 2.69 years) was not significantly different from that of uninfected ones (4.21 ± 2.96).

In all those samples from which sequencing was performed, *H. canis* was identified, with 99–100% sequence similarities to various GenBank references (mostly from Croatia and Spain). In the Hungarian dog samples three




| designation | nucleotide position (18S rRNA gene of *H. canis*) | | | | | |
|---|---|---|---|---|---|---|
| | 208 | 210 | 640 | 660 | 782 | 783 |
| genotype-A | G | T | G | T | T | G |
| genotype-B | • | • | • | • | • | A |
| genotype-C | A | G | A | G | C | A |

**Fig. 1.** Genetic variations (genotypes) in the partial sequence of the 18S rRNA gene of Hungarian *H. canis* isolates, and their distribution according to sampling sites in south Hungary. Full circles indicate places with PCR-positive dogs. Genotypes found in a location are indicated by capital letters.

genotypes could be distinguished (Fig. 1), differing in their nucleotides in one or six positions (0.15 or 0.9% difference, respectively; genotype-A identical to AY150067).

### 3.2. Evaluation of tick samples

Thirty-four shepherd dogs had tick-infestation: 18 with only *Dermacentor* larvae and nymphs (up to 45 individuals per host), 15 with *Haemaphysalis concinna* larvae and nymphs, and one dog with both species (Table 1). Larvae and nymphs of *Dermacentor marginatus* predominated among *Dermacentor* specimens (86%, CI 71–95%) which were more frequently found on PCR-positive animals (on 11 out of 19), than those of *Ha. concinna* (on 3 out of 16). This was a significant association ($P = 0.036$). Consequently, from the molecularly evaluated *Dermacentor* larvae and nymphs (55 specimens) 37 tested positive and 18 negative in the *Hepatozoon* PCR, whereas for *Ha. concinna* (28 specimens) this proportion was only 9 positive vs. 19 negative, meaning also a significant ($P = 0.004$) difference between the two tick species.

Altogether the PCR status (positivity or negativity) of both tick species reflected that of the dog from which they had been removed, i.e., all except two evaluated specimens collected from parasitaemic dogs were also *Hepatozoon*-positive (Table 1). On the other hand, a PCR-negative dog harboured PCR-positive *Ha. concinna* nymphs (Table 1).

In summary, 46 ticks (out of 83) turned out to be PCR-positive, including three *Dermacentor* larvae. Sequences obtained from these samples (for *Dermacentor*: genotypes A and B, for *Haemaphysalis*: genotypes B and C: Table 1) showed 99–100% similarity to those of *H. canis* already deposited in the GenBank. Sequences of *H. canis* found in *Dermacentor* larvae and nymphs always corresponded to those in the blood of their host, but for *Ha. concinna* different genotypes were found in ticks and their hosts, and also in ticks collected from the same dog (Table 1).

## 4. Discussion

These results provide the first evidence of *H. canis* in Hungary. This is also the first report on the endemicity of canine hepatozoonosis in any European region north of Mediterranean and Balkan countries, where *R. sanguineus* is not known to occur. In the present study canine hepatozoonosis had significantly higher prevalence than the mean prevalence (11.8%) in Croatia, or the local prevalence rates within 200 km south of Hungary where *R. sanguineus* is endemic (Vojta et al., 2009).

The northernmost limit for the geographical range of *R. sanguineus* in Central Europe is thought to be south of the Hungarian border in Croatia, Serbia and Romania (Estrada-Peña et al., 2013). In studies on canine hepatozoonosis from Mediterranean regions *R. sanguineus* is always found in association with dogs (Gabrielli et al., 2010; Otranto et al., 2011; Dantas-Torres et al., 2012). However, along the south-western border of Hungary evaluated in the present study – also taking into account seasonality – no *Rhipicephalus* spp. were found in recent large scale studies: neither among questing ticks (Hornok and Farkas, 2009), or ticks of ruminants (Hornok et al., 2012) and carnivores including shepherd dogs (Hornok et al., 2013). Rare encounters with *R. sanguineus* in Hungary were explained by accidental imports of ticks from the south (Babos, 1965; Hornok and Farkas, 2005). Further on, not a single specimen of *R. sanguineus* was found on dogs of the present study. These data confirm, that *R. sanguineus* is not endemic in southern Hungary.

Consequently, there should be factors in the background of canine hepatozoonosis described here, that are independent of *R. sanguineus*. One such factor could be the vertical transmission of *H. canis* as already reported (Murata et al.,

**Table 1**
Comparative Hepatozoon-PCR and sequencing results of dogs and their ticks. Full circles represent PCR-positive ticks; empty circles stand for PCR-negative ticks. For legends of genotypes see Fig. 1.

| | | Number of dogs (*H. canis* genotype) | Number and PCR status of removed ticks (*H. canis* genotype) |
|---|---|---|---|
| PCR-positive dogs | Dogs without ticks | 19 | – |
| | Dogs with *Dermacentor* larva/nymph | 5 (A) | ● (A) |
| | | 2 | ●●● |
| | | 1 (B) | ●●●● (B) |
| | | 1 (B) | ●●●●● (B) |
| | | 1 (A) | ●●●●○ (A) |
| | | 1 (B) | ●●●●●●●●●●●● (B) |
| | Dogs with *Haemaphysalis* larva/nymph | 1 (C) | ○ |
| | | 1 (C) | ●● (B) |
| | | 1 (C) | ●●●● (B) ● (C) |
| PCR-negative dogs | Dogs without ticks | 73 | – |
| | Dogs with *Dermacentor* larva/nymph | 4 | ○ |
| | | 2 | ○○ |
| | | 1 | ○○○○○○○ |
| | Dogs with *Haemaphysalis* larva/nymph | 7 | ○ |
| | | 3 | ○○ |
| | | 1 | ○○○ |
| | | 1 | ●●○ |
| | Dog with mixed infestation | 1 | ○○○ |

1993), explaining why several young puppies were shown to be infected during the present study. However, this mode of infection alone may not be responsible for the unexpectedly high prevalence. There must be additional epidemiological factor(s) that are most likely related to the habits and habitats of shepherd dogs.

First, for any dog the main route of infection with *H. canis* is the ingestion of adult *R. sanguineus* containing oocysts (Baneth et al., 2003), either during grooming or by eating tick-carrier small mammals. Theoretically, *H. canis* "tissue cysts" (Baneth and Shkap, 2003) in wild animal reservoirs (including carnivores and their carrion, perhaps rodents) – which may be eaten much more frequently by shepherd dogs, than pet dogs – may be infective, as proven for *H. americanum* (Johnson et al., 2008), and thus predation would also be an alternative mode of transmission (Johnson et al., 2009) in case of *H. canis*. Supporting the epidemiological role of wild carnivores (i.e. access to their carrion) in the present findings, the two most significant species, the red fox (*Vulpes vulpes*) and the golden jackal (*Canis aureus*) are more densely populated west of than east of the Danube river (Heltai and Szemethy, 2005); and the latter species is especially abundant in Somogy and Baranya counties (Szabó et al., 2009) where the great majority of *H. canis*-infections were diagnosed in the present study. In addition, visitation of small mammal (rodent) nests or burrows of foxes by PCR-positive shepherd dogs of the present study is well indicated by the fact that a high portion of these animals was infested with *Dermacentor* nymphs, known for their nidiculous (nest-associated) habit (Meyer-König et al., 2001).

Secondly, vectors other than *R. sanguineus* may be involved in the transmission. *H. canis* was already detected in questing individuals of *Ixodes ricinus* (Gabrielli et al., 2010; Reye et al., 2010), and in *Haemaphysalis* species of Japan (Murata et al., 1995). The present results indicate that *Dermacentor* spp. and *Ha. concinna* nymphs at least may become infected with *H. canis*. This is further supported by the observations that in case of *Ha. concinna* (1) PCR-positive nymphs were also found on a PCR-negative dog, and (2) there were differences between the genotypes of ticks and their hosts. Furthermore, here all except one *Dermacentor* nymphs collected from PCR-positive dogs were also PCR-positive (i.e. they did not digest all gamonts).

*R. sanguineus*, the most important vector of *H. canis* becomes infected in the nymph stage, and will contain oocysts of the protozoan at the adult stage. The great majority of *Dermacentor* and *Haemaphysalis* specimens (including PCR-positive ones) in the present study were nymphs, and this raises the question for future studies, if these ticks can be competent vectors, i.e. if they may harbour *H. canis* oocysts after moulting to the adult stage. If so, it would provide yet another clue why shepherd dogs are so frequently *Hepatozoon*-infected. In the present study *D. marginatus* nymphs were far the most prevalent ticks on shepherd dogs. However, adults of this species (perhaps the source of infection with *H. canis*) are very rare on pet dogs and in their environment (Földvári and Farkas, 2005), as contrasted to shepherd dogs which have permanent access to *D. marginatus* from livestock animals.

In light of the present results epidemiological factors in canine hepatozoonosis should be reconsidered, because the occurrence of *H. canis* in Europe north of the Mediterraneum – in Slovakia (Majláthová et al., 2007), Ireland (Maguire et al., 2010) and Poland (EU165370) – may not be attributable to accidental import of *R. sanguineus*, as previously thought.

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